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Methionine and leucine requirement and their biosynthetic blockage in C. elegans

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METHIONINE AND LEUCINE REQUIREMENT AND THEIR BIOSYNTHETIC BLOCKAGE IN *C. ELEGANS*

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Wei-chen Feng

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INFORMATION TO USERS

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ABSTRACT

METHIONINE AND LEUCINE REQUIREMENT AND THEIR BIOSYNTHETIC BLOCKAGE IN *C. ELEGANS*

by Wei-chen Feng

Caenorhabditis elegans were cultivated in a chemically defined media with six concentrations of methionine (0.0, 0.097, 0.19, 0.39, 0.78 and 1.6 mg/ml) and leucine (0.0, 0.36, 0.72, 1.4, 2.9 and 5.8 mg/ml). Optimal population growth occurred at 0.19 and 0.39 mg methionine/ml of medium and 0.72, 1.4 and 2.9 mg leucine/ml of medium. In the methionine precursor (homoserine, O-succinyl-homoserine, cystathionine, DL-homocysteine, D and L-homocysteine thiolactone·HCl) experiments, the results indicated that L and D-homocysteine thiolactone·HCl showed 46% and 9.3% efficiency as methionine replacements. In the leucine precursor (α-ketoisovaleric acid, α-isopropylmalic acid and α-ketoisocaproic acid) experiments, the results indicated that leucine precursors tested were not an effective leucine substitute. Therefore, it was concluded that the final blockage step for methionine occurred between cystathionine and homocysteine and the final blockage step for leucine occurred between α-ketoisocaproic acid and leucine.

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PREFACE

The following is a publication style thesis. The second chapter is written in journal format according to 2003 guidelines and will be submitted to Experimental Biology and Medicine. Chapters 1 and 3 are written according to guidelines outlined in the Publication Manual of the American Psychological Association (4^{th} edition), 1994.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Caenorhabditis elegans (*C. elegans*) is a small egg-laying microscopic nematode. It has a small body size (1.0 to 1.2 millimeter (mm) in length and 0.05 to 0.07 mm in diameter), a short life cycle (20 to 30 days) and a rapid generation time (Zuckerman, 1980). It exists in two sexual forms - hermaphrodite and male. *C. elegans* has been cultivated in the laboratory under axenic (germ-free) conditions since 1948 (Nicholas, 1984). *C. elegans* can also be cultivated in a chemically defined medium for bioassay (Lu, Hugenberg, Briggs, & Stokstad, 1978). For these reasons, *C. elegans* has become widely used as an animal model in the studies of biology (Zuckerman, 1980), geriatrics (Vanfleteren & Braeckman, 1999), genetics (Walhout, Endoh, Thierry-Ming, Wong, & Vidal, 1998) and nutrition (Fatt, 1967; Lu, Cheng, & Briggs, 1983; Lu & Goetsch, 1993).

Methionine and leucine have been established as essential dietary requirements for the nematodes (Vanfleteren, 1973). However, their quantitative requirement for optimal growth and maintenance has not yet been demonstrated. Moreover, knowledge about the blockages in their biosynthetic pathways is not complete, and is necessary to completely establish the nutritional requirements of these amino acids in nematodes.

The objectives of this study were to quantitatively determine the nutritional requirements of methionine and leucine and to investigate the blockages in the biosynthetic pathways of these two essential amino acids in *C. elegans*.

Review of Literature

Nematode

Biological Characteristics

Caenorhabditis elegans (*C. elegans*) is a small egg-laying, self-fertilizing and free-living soil nematode. It has a small cylindrical shaped body (Figure 1). An adult nematode is approximately 1.0 to 1.2 millimeter (mm) in length and 0.05 to 0.07 mm in diameter (Vanfleteren & Braeckman, 1999). *C. elegans* consists of approximately 10^3 somatic cells, in comparison to humans who have 10^{14} cells (Marx, 1984; Heyningen, 1997). The life cycle of *C. elegans* is approximately 20 to 30 days. Its average generation time is 3.5 days at an incubation temperature of 20°C (Zuckerman, 1980). The growth and reproduction of *C. elegans* is very sensitive to temperature. Croll and Mathews (1977) reported that the optimal population growth of *C. elegans* could be achieved at an incubation temperature of 18° to 22°C in an axenic medium.

C. elegans exists in two forms, hermaphrodite and male. Hermaphrodites produce oocytes and sperms and can reproduce by self-fertilization. Therefore, *C. elegans* can be cloned from a single hermaphrodite. However, males also fertilize hermaphrodites, but usually grow at 0.1 to 0.2% frequencies (Vanfleteren & Braeckman, 1999; Wood, 1988). The fertilized eggs of *C. elegans* after hatching (first larval stage), proceed through three more larval stages before reaching adulthood (Creighton, 1999). The dauer larva, which can occur at the second larval stage due to unsuitable conditions (e.g., starvation) can delay maturation.

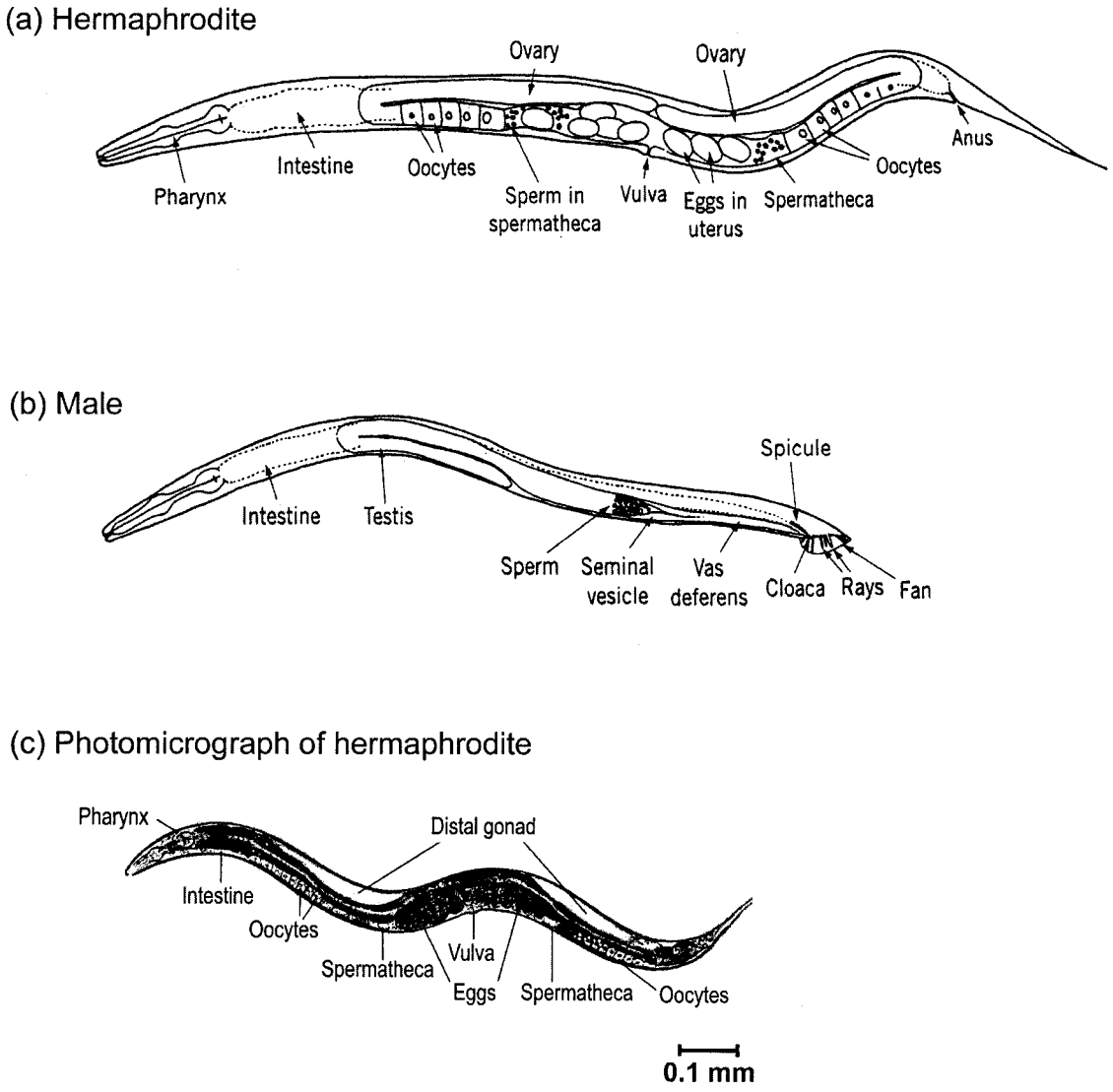


Figure 1. Anatomy of adult *Caenorhabditis elegans.* (a) Hermaphrodite; (b) Male; (c) Hermaphrodite, bright-field photomicrograph.

Note. Adapted from Encyclopedia of Molecular Biology (p. 322) by T. E. Creighton, 1999, New York: John Wiley & Sons, Inc.

Walhout et al. (1998) investigated the genome sequence of *C. elegans*. The composition of *C. elegans* genome sequence was reported to be beneficial not only to nematode research but also for the elucidation of the human gene function. *C. elegans* also provides fundamental information on its development and response to its environment (Walhout et al., 1998).

Development of Medium

The natural habitat of *C. elegans* is in the soil, with soil microorganisms as food sources. However, in the laboratory, *C. elegans* has been cultivated axenically in a chemically defined medium consisting of *C. briggsae* Maintenance Medium (CbMM) and three additional growth factors - β-sitosterol, cytochrome c (heme source) and an energy source (acetate or glucose) (Lu & Goetsch, 1993). CbMM was composed of pre-determined amounts of amino acids, vitamins, nucleic acids, minerals and other growth factors (Buecher, Hansen, & Yardwood, 1966). Later, Lu and Goetsch (1993) modified the CbMM and developed the *C. elegans* Maintenance Medium (CeMM), which included the three additional growth factors - β-sitosterol (50 μg/ml), cytochrome c (50 μg/ml) and glucose (32.5 mg/ml) or acetate (mg/ml), that supported optimal growth and development of the nematodes.

C. elegans can be axenically (germ-free) cultivated in large quantities (90,000 nematodes per ml) using the *C. elegans* Maintenance Medium (CeMM) established by Lu and Goetsch in 1993. The CeMM is an extremely rich medium, consisting of pre-determined amounts of amino acids (Table 1.1), vitamins, nucleic acids, minerals, glucose, cytochrome c and β-sitosterol (see Appendix).

Table 1.1

Amino Acid Components in CeMM

Essential Amino Acids (mg/ml)		**Non-essential Amino Acids** (mg/ml)	
L-Arginine	0.9750	L-Tyrosine	0.2720
L-Histidine	0.2830	L-Alanine	1.3950
L-Lysine·HCl	1.2830	L-Aspartic Acid	1.6200
L-Tryptophan	0.1840	L-Cysteine·HCl·H_2O	0.0280
L-Methionine	0.3890	L-Glutamate (Na)·H_2O	0.5500
L-Threonine	0.7170	L-Glutamine	1.4630
L-Leucine	1.4390	L-Glycine	0.7220
L-Isoleucine	0.8610	L-Proline	0.6530
L-Valine	1.0200	L-Serine	0.7880
L-Phenylalanine	0.8030		

Nutritional Requirements of *C. elegans*

The nutritional requirements of *C. briggsae* and *C. elegans* have been extensively studied. They have become important model systems for biological and nutritional studies since 1953 (Friedman, Platzer, & Eby, 1977). These nematodes are closely related species and differ only in genetic composition and the ability to interbreed. The hermaphrodites of both species cannot be distinguished from one another by simple observation. In 1977, Friedman, Platzer, and Eby used electrophoretic analysis of malate dehydrogenase and proteins to differentiate *C. briggsae* and *C. elegans*. It was found that some papers published prior to 1977 on *C. briggsae* might have in fact described work on *C. elegans*.

Vitamins. In 1967, Fatt reported highest *C. elegans* populations at 23°C incubation temperature. The experimental medium contained 80% liver extract, 20% peptone salt with a 5-fold concentration of thiamin, folic acid and riboflavin. It was concluded that proper mineral balance and vitamins were essential requirements for optimal growth of *C. elegans*. Lu, Hieb, and Stokstad (1974) demonstrated that in *C. briggsae*, folic acid was required for the catabolism of histidine. Since formimino L-glutamic acid accumulated in the nematode tissue when folic acid was deficient, it was concluded that folic acid was essential for histidine catabolism in *C. briggsae*. Later, Lu, Hieb, and Stokstad (1976) reported that the biosynthesis of methionine from homocysteine in *C. briggsae* required supraoptimal concentrations of both vitamin B_{12} (3.75 μg/ml) and folic acid (2 μg/ml).

The effects of all three forms of vitamin B_6 (pyridoxamine, pyridoxine, and

pyridoxal phosphate) on the growth-promoting activity of *C. elegans* were quantitatively determined by Sun et al. (1986). It was concluded that optimal level of pyridoxal phosphate (1.5×10^{-2} n mole/ml) generated the greatest growth-promoting activity, while also exhibiting the highest toxicity according to population growth. Vitamin B_6 deficiency level of all three forms was determined to be 1.5×10^{-5} n mole/ml and was noted to cause tryptophan metabolites (xanthurenic acid and kynurenic acid) to accumulate in the nematode culture medium.

Thiamin requirement was determined in the axenic culture of free-living nematodes, *C. elegans*, with two different energy sources (glucose and acetate) and five levels of thiamin (0, 0.0075, 0.075, 0.75, and 7.5 μg/ml) by Augustin et al. in 1994. It was reported that thiamin at concentrations of 0.075 to 7.5 μg/ml supported optimal population growth in *C. elegans*. And, it was concluded that a higher thiamin concentration (7.5 μg/ml) was required when acetate was used as an energy source, whereas when glucose was used as the energy source, only 0.75 μg thiamin per ml was required. This might be because, in the absence of glucose as an energy substrate, *C. elegans* relies primarily on acetate for energy production via the glyoxylate and tricarboxylic acid (TCA) cycles. The glyoxylate cycle bypasses acetyl-CoA from the TCA cycle and synthesizes four-carbon compounds (malate, oxaloacetate) from two-carbon units (acetyl-CoA, glyoxylate), instead of continued oxidation to carbon dioxide and water by the TCA cycle. The glyoxylate cycle begins with acetate to produce malate, which ultimately is converted to pyruvate via oxaloacetate and phosphoenolpyruvate as intermediates. Therefore, in a culture medium containing acetate as the sole energy

source, during thiamin deficiency, it is possible that this metabolic pathway would result in additional accumulation of pyruvate in nematode tissues and culture media. However, there was no significant difference in population between 0.075 to 7.5 μg/ml thiamin for nematodes cultivated in either glucose or acetate media. Therefore, it was suggested that lactate to pyruvate (L:P) ratio could be used to assess thiamin status in *C. elegans* (Augustin et al., 1994).

Li et al. (1995) studied the effect of different concentrations of nicotinic acid, nicotinamide, and nicotinic acid plus nicotinamide on the population growth of *C. elegans*. It was reported that nicotinic acid demonstrated greater growth-promoting activity than nicotinamide at all concentrations, indicating that nicotinic acid is the more active form than nicotinamide in the nematodes. Population growth supported by media containing both nicotinic acid and nicotinamide was slightly greater than the growth supported by nicotinic acid or nicotinamide alone. Nicotinic acid plus nicotinamide concentrations between 0.30 to 38 μg/ml each promoted optimal population growth of *C. elegans*. It was also determined that the conversion ratio of tryptophan to niacin is 500:1 in *C. elegans*, compared to 60:1 in humans (Whitney, Cataldo, & Rolfes, 1994).

Minerals. Lu, Cheng, and Briggs (1983) reported that magnesium (73 μg/ml), sodium (300 μg/ml), potassium (530 μg/ml), manganese (6.3 μg/ml), calcium (1500 μg/ml) and copper (7.2 μg/ml) were required by *C. briggsae*. Weber and Lu (1992) later reported that zinc at concentrations ranging between 4.9 to 37 μg/ml supported optimal population growth in *C. elegans*.

Amino acids. Vanfleteren (1973) investigated the amino acid requirements for nematodes. *C. briggsae* could not synthesize the following amino acids: arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine. These amino acids were therefore determined to be dietary essential amino acids. On the other hand, alanine, asparagine, cysteine, glutamate, glutamine, glycine, proline, serine and tyrosine were synthesized by *C. briggsae*. These were referred to as dietary nonessential amino acids. The quantitative levels for each of these amino acids were not determined. Perelman and Lu (2000) reported that branched-chain amino acids (valine, leucine, isoleucine) were required for the growth and reproduction of *C. elegans*. Optimal population growth for *C. elegans* was achieved when leucine was supplemented in the CeMM at the concentrations of 0.72 to 2.8 mg/ml, isoleucine at concentrations of 0.86 to 1.7 mg/ml, and valine at concentrations of 0.51 to 4.1 mg/ml.

Other Growth factors. Hieb and Rothstein (1968) reported that sterol was required for the reproduction of *C. briggsae*. A single sterol or a mixture of sterol compounds can satisfy the sterol requirement of *C. elegans*. Sterol mixture at the concentration of 1.3 μg/ml supported optimal population growth in *C. briggsae*. Lu, Newton, and Stokstad (1977) reported that β-sitosterol at a concentration of 50 μg/ml, supported optimal population growth in the nematodes - *C. briggsae*, *C. elegans*, and *Turbatrix aceti*. In 1978, Lu et al. reported that other lipid-related compounds - tween 80, tween 85, sodium oleate, sodium stearate, ethanol, n-propanol, and potassium acetate were also required for the growth of *C. briggsae*.

C. briggsae also requires heme, which is an iron-containing porphyrin compound (Hieb, Stokstad, & Rothstein, 1970). Result from this study indicated that cytochrome c is one of the most effective heme source for growth and reproduction in *C. briggsae*. Chang et al. (1988) investigated the effect of six different concentrations of cytochrome c and three surface area exposures of the medium on the population growth and ATP production of *C. elegans*. It was concluded that the basal medium supplemented with cytochrome c at a concentration of 200 μg/ml and a surface area exposure of 12 cm^2 per 5 ml medium supported the optimal population growth in *C. elegans*.

Energy source. In CbMM, *C. elegans* utilized either potassium acetate or other lipid related factors as an energy source (Lu, Hugenberg, Briggs, & Stokstad, 1978). In CeMM, *C. elegans* could also utilize carbohydrate as a major energy source. Glucose (32.5 mg/ml) was demonstrated to support the maximal population growth in nematodes (Lu & Goetsch, 1993).

Methionine

Metabolic Roles of Methionine

Methionine is an essential amino acid for mammals and is synthesized de novo in most bacteria, fungi and plants (Bright, Lea, & Miflin, 1980). Methionine is the only sulfur-containing essential amino acid for mammals and it must be provided from the diet (Ravanel, Gakiere, Job, & Douce, 1998). Methionine is essential for the biosynthesis of a variety of cellular components including creatine, epinephrine, carnitine, phospholipids, protein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It also holds essential metabolic functions in the synthesis of S-adenosylmethionine (SAM) and homocysteine

(Finkelstein, 2000).

Methionine is activated by ATP to form S-adenosylmethionine (SAM), in a reaction catalyzed by the enzyme, S-adenosylmethionine synthetase. In this reaction, a sulfonium bond is formed between the 5'-carbon atom of the ribose and the sulfur atom of the amino acid. SAM is a potent methylating agent (methyl donor) in biological systems (Weissbach & Brot, 1991). In a transmethylation reaction, SAM donates a methyl group to methyl acceptors and itself becomes S-adenosylhomocysteine (SAH) (van der Put, van Straaten, Trijbels, & Blom, 2001). S-Adenosylhomocysteine hydrolase cleaves the thioether in SAH to form homocysteine and adenosine (Figure 2).

Homocysteine is a precursor of methionine in plants and bacteria (Bright, Lea, & Miflin, 1980). Homocysteine has been reported to have three different forms: (a) reduced form, (b) thiolactone form and (c) disulfide form (oxidized form) (Figure 3). Djurhuus et al. (1988) reported that homocysteine in its (a) reduced form (sulfhydryl-containing homocysteine) was highly toxic to all cells. Lu, Hieb, and Stokstad (1976) reported that methionine can be replaced by (b) homocysteine thiolactone in the *C. briggsae* Maintenance Medium (CbMM), when cultivated in the nematode, *Caenorhabditis briggsae* (*C. briggsae*). The homocysteine thiolactone was also demonstrated to support growth of non-transformed and malignant cells in a methionine deficient medium in mouse embryo fibroblasts. Later, Djurhuus and Ueland (1989) also reported that mouse fibroblasts were able to grow in homocysteine thiolactone in a methionine free medium. The thiolactone form and the (c) disulfide form of homocysteine were reported to be non-toxic in their study.

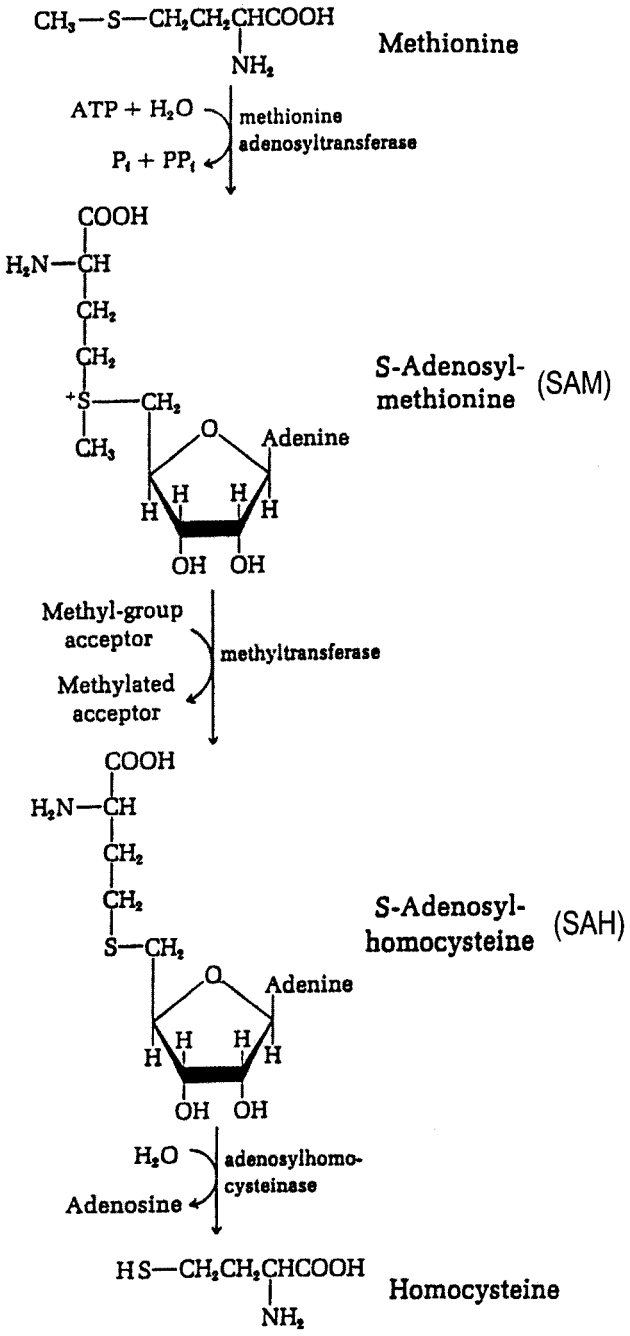
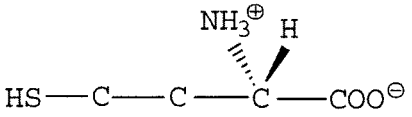


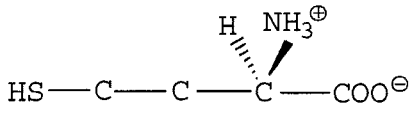
Figure 2. The conversion of methionine to SAM and SAH.

Note. Adapted from Biochemistry (p. 698) by A.L. Lehninger, 1975, New York: Worth Publishers.

(a) Reduced form of homocysteine (L and D form)

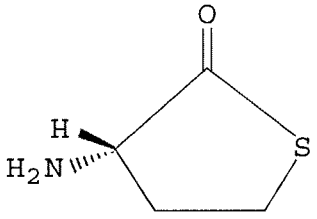


L-Homocysteine

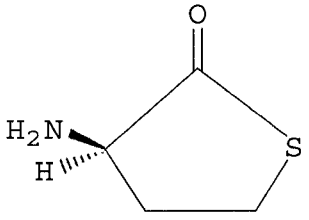


D-Homocysteine

(b) Homocysteine thiolactone (L and D form)

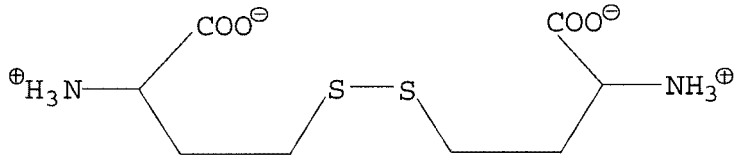


L-Homocysteine thiolactone



D-Homocysteine thiolactone

(c) Homocysteine disulfide (Oxidized form)



Homocysteine disulfide

Figure 3. Chemical structures of three forms of homocysteine. (a) reduced form of L and D-homocysteine; (b) L and D-homocysteine thiolactone; (c) homocysteine disulfide (oxidized form).

Many studies were done with methionine and its precursor, homocysteine. In 1974, Halpern et al. discovered that methionine could be replaced by L-homocysteine as an essential nutrient in the medium of growing mammalian cells, when the medium contained both folic acid and vitamin B_{12}. Halpern et al. (1974) suggested that the cells had the capacity to methylate homocysteine to methionine with 5-methyltetrahydrofolate homocysteine methyltransferase activity. Lu, Hieb, and Stokstad (1976) cultivated the nematode, *Caenorhabditis briggsae* (*C. briggsae*) in the *C. briggsae* Maintenance Medium (CbMM) containing DL-homocysteine thiolactone·HCl as methionine replacement, in the presence of vitamin B_{12} and folic acid. At supraoptimal concentrations of vitamin B_{12} (3.75 μg/ml) and folic acid (2 μg/ml), homocysteine was demonstrated to replace methionine. It was concluded that *C. briggsae* possessed a mechanism for the biosynthesis of methionine from homocysteine. However, the quantitative requirement of methionine for optimal population growth of *C. briggsae* was not determined. Baker and Czarnecki (1985) investigated the growth response of young chicks and rats and determined the efficiency of L-homocysteine thiolactone·HCl as a methionine replacement in their diet. It was reported that L-homocysteine thiolactone·HCl was 64.5% efficient in rats and 62.5% efficient in chicks, in replacing L-methionine in the diet. A higher dose of L-homocysteine thiolactone·HCl and betaine indicated enhanced conversion of homocysteine to methionine.

Nutritional Requirement of Methionine in Different Species

In a human diet, containing 10 to 15% protein, methionine is at an adequate concentration of 1.9% of the dietary protein (Scott, 1986). Animal studies indicate that

methionine is essential for dogs, swine, turkeys, rats and other species. The requirements of methionine for different animals are summarized in Table 1.2.

Pathway of Methionine Biosynthesis

The biosynthetic pathway of methionine has been studied in many organisms. The intermediates of methionine in the biosynthetic pathway are homoserine, O-succinyl-homoserine, cystathionine, and homocysteine (Lehninger, 1975) (Figure 4). The biosynthesis of methionine from homoserine begins with the enzymatic (homoserine acyltransferase) conversion to O-succinyl-homoserine. In this reaction, the succinyl group of succinyl-CoA is transferred to homoserine. Next, succinate is displaced from O-succinyl-homoserine by cysteine in the presence of the enzyme cystathionine γ-synthase, forming cystathionine (Herrmann & Somerville, 1983). Cystathionine is hydrolyzed and cleaved to yield homocysteine, pyruvate and ammonia (NH_3) by cystathionine β-lyase. In the final reaction, homocysteine is methylated to methionine by the enzyme methyl transferase. This reaction occurs in the presence of a cobalamin-dependent methionine synthase, that contains a vitamin B_{12} cofactor (Ravanel, Gakiere, Job, & Douce, 1998). Methyl transferase catalyses the transfer of a methyl group from N5-methyl-tetrahydrofolate (N5-Methyl-FH_4) to methionine. Finally, a methyl group is donated by N5-Methyl-FH_4 to homocysteine, to form methionine (Lehninger, 1975).

Table 1.2

Methionine Requirements of Different Species

Species	% Protein in diet	Requirement (mg amino acid/g protein)	Criteria used
Human Adult[1, a]	10-15	17	Maintenance
Dog Adult[1, a]	9	17	Maintenance
Swine Adult[1, a]	12	19	Maintenance
Turkey (0-4 wk)[1, a]	28	38	Optimal growth
Rat (wealing)[1]	12	50	Optimal growth
C. elegans[b]	28	25	Optimal growth
N. glaseri[2, c]	65	26	Optimal growth

[1]McLarney, Pellett, and Young, 1996.

[2]Jackson, 1973.

[a]includes cysteine.

[b]methionine in *C. elegans* Maintenance Medium.

[c]methionine in Chemically Defined Medium for *Neoaplectana glaseri.*

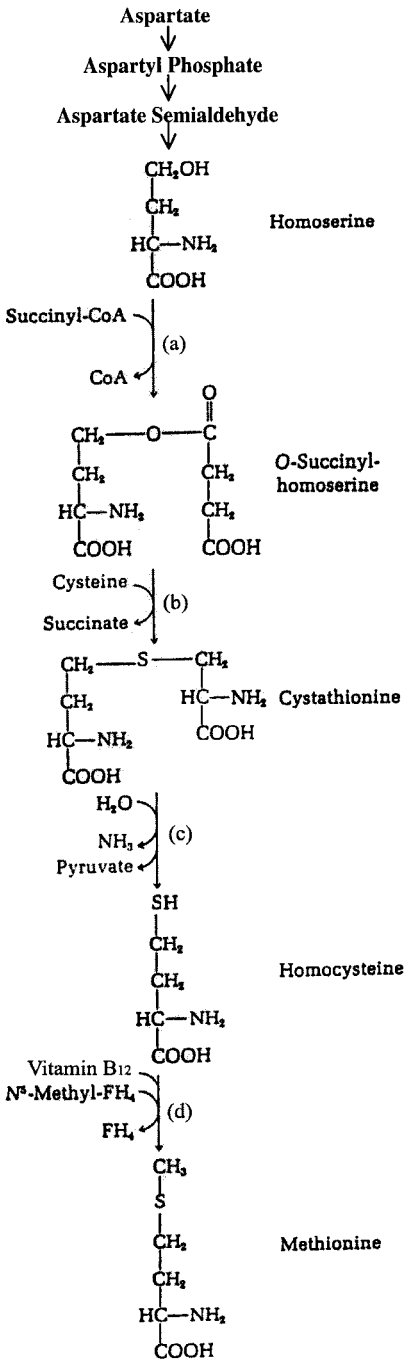


Figure 4. The biosynthetic pathway of methionine. (a) Homoserine acyltransferase; (b) Cystathionine γ-synthase; (c) Cystathionine β-lyase; (d) Methyl transferase.

Note. Adapted from Biochemistry (p.701) by A.L. Lehninger, 1975, New York: Worth Publishers.

Leucine

Metabolic roles of leucine

The branched chain amino acids (BCAAs) - leucine, isoleucine and valine are the three essential amino acids for the nematode (Vanfleteren, 1973). All three BCAAs cannot be synthesized by mammals and are degraded in tissues throughout the body. Leucine plays many regulatory roles in metabolism. It regulates protein turnover, increases protein synthesis and decreases protein degradation. Furthermore, leucine's immediate precursor α-ketoisocaproate (α-ketoisocaproic acid, KIC) inhibits proteolysis (protein breakdown), stimulates protein synthesis, and reduces the rate of protein degradation in skeletal muscle (Bender, 1985). Perelman and Lu (2000) reported that BCAAs were dietary essential amino acids for *C. elegans.* Optimal population growth was achieved when leucine was supplemented in the *C. elegans* Maintenance Medium (CeMM) at the concentration of 0.72, 1.4 and 2.8 mg/ml of the medium. The biosynthetic pathway from α-ketoisovaleric acid to leucine existed in fungi and bacteria (Lehninger, 1975). The biosynthetic blockages in the pathway were however, not demonstrated in the study.

Many research studies have been done on the biosynthetic pathway of leucine from its immediate precursor KIC. Chawla et al. (1975) reported that the addition of KIC to a leucine free diet prevented weight loss in growing rats. The efficiency of the substitution ranged between 20 to 27%. Trigg et al. (1975) reported that leucine-starved mice regained lost weight by being placed on a KIC supplemented diet. Later, Flakoll et al. (1991) found that KIC injections to growing lambs showed increased weight gain and

muscle growth and decreased fat deposition. It was concluded that KIC administration stimulated sheep growth by affecting protein metabolism, such that energy partitioning between fat and lean tissue was altered.

Conversely, Kang and Walser (1985) reported that feeding α-ketoisocaproic acid (KIC) as leucine substitute at varying dosages did not support the whole-body protein ratio and growth rate in rats. However, it was suggested that KIC could be used to replace leucine without any adverse effects, by providing the rats several days of adaptation. Kang, Tungsanga, and Walser (1986) demonstrated the nutritional efficiency of the keto acid as a substitute for leucine in rat's diet. It was found that rats fed 6% protein diet exhibited lower weight gain and feed efficiency than rats fed at higher protein intake (12, 24, and 48%). Therefore, it was concluded that the nutritional efficiency of KIC as a leucine dietary substitute was strongly dependent on the protein intake of the rat. Therefore, when protein content in diet increases, so does keto acid activity in liver and muscles. Shambaugh III and Koehler (1983) reported that KIC also had no effect on the conversion of the keto acid to leucine in fetal rat brain. They stated that the exogenous conversion of KIC to leucine in fetal rat brain depends on the nutrition status of the mother. Beyer, Jensen, and Villegas (1992) reported that KIC had no effect on growth or abdominal fat deposition in chickens. Gatnau et al. (1995) also demonstrated that KIC did not affect growth and immune response and that a high concentration of leucine injection was detrimental to growth and the immune response system in pigs.

Nutritional Requirement of Leucine in Different Species

In human diets containing 10 to 15 % protein, leucine is at an adequate level of 19 mg amino acid per gram protein (McLarney, Pellett, & Young, 1996). In animal studies, leucine was also found to be essential for dogs, swine, turkeys, rats and other species. The requirements of leucine for different animals are summarized in Table 1.3.

Table 1.3

Leucine Requirements of Different Species

Species	% Protein in diet	Requirement (mg amino acid/g protein)	Criteria used
Human Adult[1, a]	10-15	19	Maintenance
Dog Adult[1, a]	9	49	Maintenance
Swine Adult[1, a]	12	25	Maintenance
Turkey (0-4 wk)[1, a]	28	68	Optimal growth
Rat (wealing)[1]	12	63	Optimal growth
C. elegans[a]	28	93	Optimal growth
N. glaseri[2, b]	65	129	Optimal growth

[1]McLarney, Pellett, and Young, 1996.

[2]Jackson, 1973.

[a]leucine in *C. elegans* Maintenance Medium.

[b]leucine in Chemically Defined Medium for *Neoaplectana glaseri.*

The Pathway of Leucine Biosynthesis

The biosynthetic pathway of leucine begins with the condensation of α-ketoisovaleric acid and acetyl-CoA to yield α-isopropylmalic acid (Figure 5) (Lehninger, 1975). The enzyme isopropylmalate synthase catalyses this reaction and is sensitive to feedback inhibition by leucine. Next, α-isopropylmalic acid isomerises to β-isopropylmalic acid in the presence of α- isopropylmalate dehydratase (Bender, 1985). A NAD^+-dependent oxidative decarboxylation (isopropylmalate dehydrogenase) of β-isopropylmalic acid yields α-ketoisocaproic acid (KIC). Finally, KIC in the presence of glutamate is transaminated to leucine.

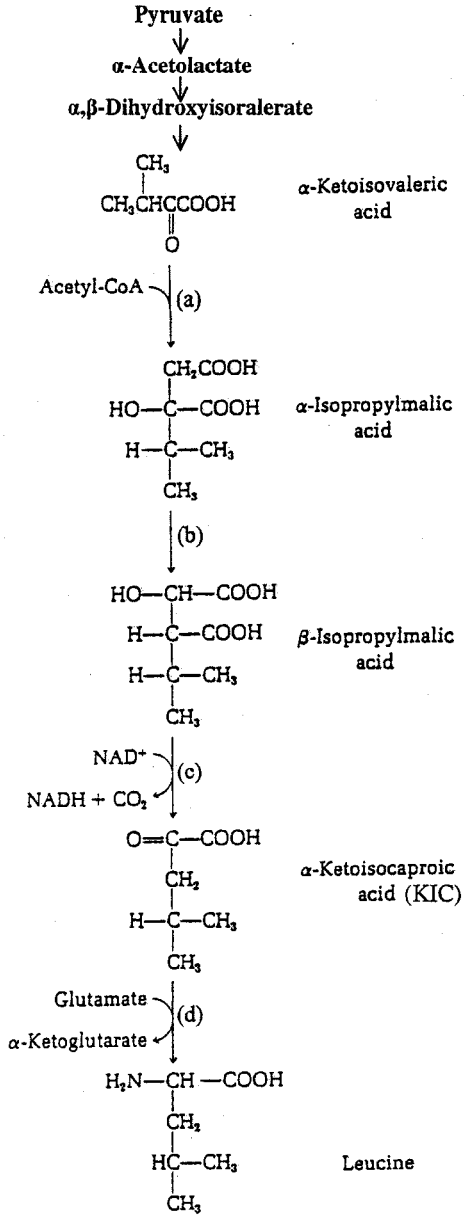


Figure 5. The biosynthetic pathway of Leucine. (a) α-Isopropylmalate synthase; (b) α-Isopropylmalate dehydratase; (c) β-Isopropylmalate dehydrogenase; (d) leucine transaminase.

Note. Adapted from Biochemistry (p.705) by A.L. Lehninger, 1975, New York: Worth Publishers.

CHAPTER 2

JOURNAL ARTICLE

Author's Title Page

METHIONINE AND LEUCINE REQUIREMENTS AND THEIR BIOSYNTHETIC BLOCKAGES IN *CAENORHABDITIS ELEGANS*

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ABSTRACT

The requirements of methionine and leucine were determined in the free-living nematode, *Caenorhabditis elegans* (*C. elegans*). The growth-promoting activities of six different concentrations of methionine (0.0, 0.097, 0.19, 0.39, 0.78 and 1.6 mg/ml) and leucine (0.0, 0.36, 0.72, 1.4, 2.9 and 5.8 mg/ml) were determined. Optimal population growth of *C. elegans* occurred at concentrations of 0.19 and 0.39 mg methionine per ml of medium. Optimal population growth of *C. elegans* occurred at concentrations of 0.72, 1.4 and 2.9 mg leucine per ml of medium. The site of blockage in the biosynthetic pathway of methionine and leucine was determined by supplementing the precursors of these amino acids in equimolar concentrations of the optimal concentration established earlier. It was concluded that the metabolic blockage(s) for methionine synthesis occurred between homoserine and homocysteine. Moreover, since L-homocysteine thiolactone·HCl had 46% efficiency and D-homocysteine thiolactone·HCl had about 9.3% efficiency as methionine substitutes, the final blockage point of methionine synthesis may have occurred between cystathionine and homocysteine. The metabolic blockage(s) of leucine occurred between α-ketoisovaleric acid and leucine, since none of the precursors tested supported growth in the nematodes.

Key words: germ-free nematode; methionine; leucine; homocysteine; precursor; culture medium.

INTRODUCTION

The free-living nematode, *Caenorhabditis elegans* (*C. elegans*) has been used as an animal model since 1948 (1). It can be cultivated in the laboratory under axenic (germ-free) conditions using a chemically defined medium (2-3). *C. elegans* not only has a short life cycle (20-30 days), but also a rapid generation time (3.5 days at 20°C) (4). The nutritional requirements of this nematode are highly similar to higher animals and microorganisms, including requirements of essential amino acids (5), vitamins (6) and minerals (7). Thus, it has become widely used as an animal model for nutrition research (5-9).

While it is known that methionine and leucine are required by the nematode, *C. elegans* (5), the quantitative requirements of methionine necessary for optimal population growth and maintenance, as well as the metabolic blockages of methionine and leucine's biosynthetic pathways are unknown. This information is necessary to complete the nutritional requirements of this animal model. *C. elegans* axenically cultured in a chemically defined media, is an excellent animal model for the investigation of metabolic pathway blockages in a multicellular organism under controlled nutritional conditions.

The objectives of this study were first to quantitatively determine the nutritional requirement of methionine and leucine individually for supporting optimal population growth of the nematode, *C. elegans* and then, to investigate the blockages in biosynthetic pathways of these two amino acids in *C. elegans*.

MATERIALS AND METHODS

Stock Media and Cultures. The experimental procedure was based on the conditions described by Lu and Goetsch in 1993 (3). The *C. elegans* stock medium contained 4% Hy Soy powder (Quest International, Norwich, NY), 1% yeast extract (DIFCO Laboratory, Detroit, MI) and 10% heated liver extract (10). *C. elegans* were first cultivated in 18 x 150 mm culture tubes in 5.0 ml of stock medium (HS-YE-HLE). Cultures were incubated at 19-20°C on a tissue culture rotator (at one rpm speed) for approximately two weeks. Nematodes from the stock culture were washed three times with deionized distilled water by centrifugation for use as innoculum in experimental cultures. Approximately 2,500 washed nematodes suspended in 0.1 ml volume were inoculated into 5.0 ml of the various experimental media, resulting in an initial population of approximately 500 nematodes per ml. The number of nematodes (nematodes/ml) was determined by counting diluted cultures under the microscope.

Experimental Media and Cultures. The experimental media (5ml/tube) consisted of CeMM (minus methionine or leucine) and six different concentrations of methionine or leucine. The media was used in determining the concentration of each amino acid necessary for the optimal population growth of *C. elegans*. The six different concentrations of methionine were 0.0, 0.097, 0.19, 0.39, 0.78 and 1.6 mg/ml. For leucine the concentrations were 0.0, 0.36, 0.72, 1.4, 2.9 and 5.8 mg/ml. CeMM (minus methionine or leucine) was prepared in double strength (2X) and was sterilized by passing the medium through a Millipore nitrocellulose filter (Millipore Corporation, Bedford, MA), with a pore diameter of 0.22 μm. Each concentration of methionine and

leucine cultures was prepared in quadruplicate. The various methionine or leucine concentrations were prepared by making four serial dilutions from the highest concentration. Then, each solution was sterilized by using the Millipore filtration method. An initial population of 520 nematodes/ml was inoculated into each culture tube (18 x 150 mm). The experimental cultures were incubated at 19-20°C on a tissue culture rotator. The population growth in each culture was counted weekly. The final population was counted on day 23, when peak population growth (about 218 x 10^3 nematodes/ml) was reached. At least two samples (0.1 ml/ea) of each experimental culture were counted under the microscope by a simple tally of individual nematodes.

Determination of Blockage(s) in the Biosynthetic Pathways of Methionine. The metabolic blockages of biosynthetic pathways of methionine were investigated by cultivating *C. elegans* in a media containing selected precursors of methionine, using the maximum growth-promoting concentrations determined in the experiments above. Methionine's precursors were added at equimolar concentration as that of methionine, at 0.39 mg/ml or 2.61 mmole.

The biosynthesis of methionine starts with aspartate and is phosphorylated and reduced in two stages, first to the aldehyde, then to alcohol, producing homoserine (11-12). Then, homoserine undergoes enzymatic conversion to O-succinyl-homoserine. In this reaction, the succinyl group is transferred to homoserine. Next, succinate is displaced from O-succinyl-homoserine by cysteine in the presence of the enzyme cystathionine γ-synthase, forming cystathionine. Cystathionine is hydrolyzed and cleaved to yield homocysteine, pyruvate and ammonia (NH_3) by cystathionine β-lyase.

In the final reaction, homocysteine is methylated to methionine by the enzyme methyl transferase. This reaction occurs in the presence of a cobalamin-dependent methionine synthase, that contains a vitamin B_{12} cofactor. Methyl transferase catalyses the transfer of a methyl group from N5-methyl-tetrahydrofolate (N5-Methyl-FH4) to methionine. Finally, a methyl group is donated by N5-Methyl-FH4 to homocysteine, to form methionine (12).

The selected precursors for methionine were homoserine (0.31 mg/ml or 2.61 mmole), O-succinyl-homoserine (0.57 mg/ml or 2.61 mmole), cystathionine (0.58 mg/ml or 2.61 mmole) and homocysteine (Sigma-Aldrich Inc., St. Louis, MO) (11). Three different forms of homocysteine were also tested: L-homocysteine thiolactone·HCl (0.40 mg/ml or 2.61 mmole), D-homocysteine thiolactone·HCl (0.40 mg/ml or 2.61 mmole) and DL-homocysteine (0.35 mg/ml or 2.61 mmole) (Sigma-Aldrich Inc., St. Louis, MO).

Each culture was prepared in quadruplicates. From the previous optimal population growth experiment, it was determined that day 23 represented the average day of the peak population growth among nematode cultures. Therefore, the final population growth was counted under the microscope on day 23.

Determination of Blockage(s) in the Biosynthetic Pathways of Leucine. The metabolic blockages of biosynthetic pathways of leucine were investigated by cultivating *C. elegans* in a media containing selected precursors of leucine, using the maximum growth-promoting concentrations determined in the experiments above. Leucine's precursors were added at equimolar concentration as that of leucine, at 1.4 mg/ml or 10.7 mmole.

The biosynthetic pathway of leucine begins with the condensation of α-ketoisovaleric acid and acetyl-CoA to yield α-isopropylmalic acid (2-isopropylmalic acid) (12). The enzyme isopropylmalate synthase catalyses this reaction and is sensitive to feedback inhibition by leucine. Next, α-isopropylmalic acid isomerises to β-isopropylmalic acid in the presence of α- isopropylmalate dehydratase. A NAD^+-dependent oxidative decarboxylation (isopropylmalate dehydrogenase) of β-isopropylmalic acid yields α-ketoisocaproic acid (KIC). Finally, KIC in the presence of glutamate, is transaminated to leucine (12).

The precursors for leucine used were α-ketoisovaleric acid (1.5 mg/ml or 10.7 mmole), α-isopropylmalic acid (1.9 mg/ml or 10.7 mmole) (2-isopropylmalic acid) and α-ketoisocaproic acid (1.7 mg/ml or 10.7 mmole) (Sigma-Aldrich Inc., St. Louis, MO) (12).

Each culture was prepared in quadruplicates. From the previous optimal population growth experiment, it was determined that day 23 represented the average day of the peak population growth among nematode cultures. Therefore, the final population growth was counted under the microscope on day 23.

Statistical Analysis. Paired simple t-test procedure was utilized to determine the statistical significance of differences in mean values of population growth of *C. elegans* at various concentrations of methionine and leucine. Statistic significant at 0.05 level. Results of methionine and leucine precursor experiments were presented as mean ± standard deviation of four culture tubes. All the data was analyzed using SPSS® (SPSS

Inc., Upper Saddle River, NJ).

RESULTS

Growth Pattern with Methionine and Leucine. Population growth of *C. elegans* as supported by six different concentrations of methionine (0.0, 0.097, 0.19, 0.39 (CeMM level), 0.78 and 1.6 mg/ml) and six different concentrations of leucine (0.0, 0.36, 0.72, 1.4 (CeMM level), 2.9 and 5.8 mg/ml) are shown in Figure 1 and 2, respectively. In this study, *C. elegans* exhibited a logarithmic growth pattern with both methionine and leucine during the first 14 days, after which the growth slowed down. The maximum population growth was attained on the 23rd day. Methionine or leucine free media did not support adequate growth. In addition, many small-sized adults and dead nematodes were observed in these media.

Quantitative Requirement of Methionine. Based on the dose response curve of methionine (Figure 1), the maximal population growth ($218 \pm 17 \times 10^3$ nematodes/ml) for *C. elegans* was achieved when methionine was supplemented in the media at concentrations of 0.19 and 0.39 mg/ml. There was no significant difference on the population growth of *C. elegans* between concentrations of 0.19 and 0.39 mg methionine per ml of medium. At concentrations of 0.0 and 0.097 mg methionine per ml of medium, the population growth ($0.20 \pm 0.12 \times 10^3$ and $155 \pm 5.3 \times 10^3$ nematodes/ml respectively) was significantly lower, indicating methionine deficiency. At high concentrations 0.78 and 1.6 mg methionine per ml of medium, the population growth was reduced to 94 ± 17 and $22 \pm 0.58 \times 10^3$ nematodes per ml respectively, indicating methionine toxicity.

Quantitative Requirement of Leucine. Based on the dose response curve of leucine (Figure 2), the optimal population growth ($218 \pm 22 \times 10^3$ nematodes/ml) for *C. elegans* was achieved when leucine was supplemented in the media at 0.72, 1.4, and 2.9 mg/ml. There were no significant differences in population growth at leucine concentrations of 0.72, 1.4 and 2.9 mg/ml in the experimental media. At leucine concentrations of 0.0 and 0.36 mg/ml, the population growth was significantly reduced ($p < .05$) from 0.0 to $92 \pm 3.3 \times 10^3$ nematodes/ml. Therefore, these concentrations were determined to be leucine deficient. At a concentration of 5.8 mg leucine per ml of medium, which was determined to be toxic to the nematodes, the population growth was reduced to $13 \pm 1.5 \times 10^3$ nematodes/ml from the maximal population growth of $218 \pm 22 \times 10^3$ nematodes/ml.

Studies on Methionine Pathway. *C. elegans* population growth in a methionine free medium with supplementation of L-homocysteine thiolactone·HCl was $93 \pm 1.9 \times 10^3$ nematodes/ml. Supplementation with D-homocysteine thiolactone·HCl yielded a population growth of $19 \pm 2.6 \times 10^3$ nematodes/ml of a methionine free medium. Both these precursors achieved much less population growth compared to a medium containing methionine ($203 \pm 13 \times 10^3$ nematodes/ml). In other words, L-homocysteine thiolactone·HCl and D-homocysteine thiolactone·HCl demonstrated 46% and 9.3% efficiency when replacing methionine in the basal medium for *C. elegans*.

Adequate population growth was not noticed with the methionine free medium containing DL-homocysteine ($1.0 \pm 0.13 \times 10^3$ nematodes/ml). The first three methionine precursors, homoserine, O-succinyl-homoserine and cystathionine in a

methionine free medium did not support the population growth of *C. elegans* as well (Table 2.1).

Studies on Leucine Pathway. All of the leucine precursors used in the experiment, α-ketoisovaleric acid , α-isopropylmalic acid (2-isopropylmalic acid), and α-ketoisocaproic acid failed to support the population growth of *C. elegans* in CeMM (minus leucine). A population growth of 100 nematodes/ml, 50 nematodes/ml and 300 nematodes/ml were attained with α-ketoisovaleric acid, 2-isopropylmalic acid and α-ketoisocaproic acid, compared to a population growth of 223 x 10^3 nematodes/ml in leucine (Table 2.2).

DISCUSSION

Optimal population growth of *C. elegans* was achieved at methionine concentrations of 0.19 and 0.39 mg/ml of medium. There were no significant differences in population growth at these concentrations. At methionine concentration below 0.19 and above 0.39 mg/ml, sub-optimal population growth was produced, indicating deficient or toxic concentrations respectively. Results therefore indicated that the optimal requirement of methionine for maximum population growth of *C. elegans* was 0.19 and 0.39 mg/ml. A concentration of 0.39 mg/ml methionine was adopted in CeMM (3), which is agreeable to the findings from our study. Moreover, methionine at 0.19 mg/ml also produced optimal growth and there was no significant difference in population growth of the nematodes between these two concentrations. Therefore, the methionine concentration in CeMM could be lowered to 0.19 mg/ml instead of 0.39 mg/ml currently in CeMM. It was established from our study that, in comparison to *C. elegans*, the

parasitic nematode *Neoaplectana glaseri* required a higher concentration (0.6 mg/ml) of methionine for optimal growth (13). Optimal population growth of *C. elegans* was achieved at leucine concentrations of 0.72, 1.4 and 2.9 mg/ml. No significant differences in population growth were observed at these concentrations. Population growth was significantly decreased ($p< .05$) at leucine concentrations of 0.36 and 5.8 mg/ml, indicating severe leucine deficiency and toxicity. The results from this study concurred with the findings of Perelman and Lu (9), who reported that *C. elegans* required a leucine concentration of 0.72, 1.4 and 2.9 mg/ml for optimal population growth in the culture media. The concentrations cover the 1.4 mg per ml currently used in the CeMM (3). Since there are no significant differences in population growth of the nematodes for the three different concentrations of leucine, the lowest concentration could be determined as the optimal concentration requirement of *C. elegans*. Therefore, the leucine concentration could also be lowered to 0.72 mg per ml in CeMM. Studies with *N. glaseri* (13) indicated a much higher leucine requirement (3.0 mg/ml) for this nematode.

Based on the results of this study, it was found that methionine can be replaced by L-homocysteine (Table 2.1) in the nematodes. Lu et al. (6) cultivated the nematode, *Caenorhabditis briggsae* (*C. briggsae*) in the *C. briggsae* Maintenance Medium (CbMM) containing double concentration of DL-homocysteine thiolactone·HCl (assuming only L form is active) as the methionine replacement. The investigators demonstrated that methionine can be replaced by homocysteine thiolactone, when supraoptimal levels of vitamin B_{12} (3.75 μg/ml) and folic acid (7.5 μg/ml) were provided. This indicates that, as in higher animals and microorganisms (6), nematodes possessed a mechanism for the

biosynthesis of methionine from homocysteine. Our results further indicated that L-homocysteine thiolactone·HCl had a greater conversion efficiency (46%) than D-homocysteine thiolactone·HCl (9.3%) on an equimolar basis. Homocysteine also proved to be a replacement for methionine in young chicks, rats (14), mammalian cell cultures (15) and mouse embryo fibroblasts (16). Our findings supported these research studies. Studies with parasitic nematode *Angiostrongylus costaricencis* (17) also indicated that growth and development was not supported with D-isomer replacements of L-amino acids. Since all natural amino acids isolated from protein have the same L configuration (18), D-isomer replacements might not be efficient as substitutes.

Our study also found that DL-homocysteine (reduced form) was not an effective substitute for methionine in *C. elegans*. Djurhuus et al. (19) reported that homocysteine in its reduced form (sulfhydryl-containing homocysteine) was highly toxic. The homocysteine thiolactone was demonstrated to support growth of non-transformed and malignant cells in a methionine deficient medium in mouse embryo fibroblasts. Later, Djurhuus and Ueland (16) also reported that mouse fibroblasts were able to grow in homocysteine thiolactone in a methionine free medium. The thiolactone form and the disulfide form of homocysteine were reported to be non-toxic in their study.

Based on the results from our study, there was no population growth noted with cystathionine. The metabolic blockage(s) in the biosynthetic pathway of methionine from homoserine might have occurred at any step(s) between homoserine and homocysteine. However, final blockage may have occurred between cystathionine and homocysteine.

Previous reports that leucine was an essential amino acid for *C. elegans* (5, 9) is further confirmed from this study. It is also shown that leucine cannot be replaced by any of the precursors tested (Table 2.2). These results strongly indicated that α-ketoisocaproic acid and other leucine precursors were not effective replacements of leucine for *C. elegans*. α-Ketoisocaproic acid (KIC) has been shown as a leucine replacement in different animal species, such as rats (20), mice (21) and lambs (22). However, our results did not support these findings. Studies with mice and rats might not have been conducted in an axenically controlled environment. Intestinal microorganisms present in these laboratory animals might also be another reason for contradictory results. Other studies with rats (23-25), broilers (26) and pigs (27) have also reported KIC as an ineffective leucine replacement, as demonstrated from our study. Therefore, it was concluded that the multiple metabolic blockages for leucine may have occurred between α-ketoisovaleric acid and leucine, but the final metabolic blockage step occurred between α-ketoisocaproic acid and leucine.

CONCLUSION

In summary, results of the present study indicated that the optimal concentration for supporting nematode population growth for methionine was achieved in the basal medium at concentrations of 0.19 and 0.39 mg/ml. There was no significant difference ($p < .05$) in the population growth at these levels. Leucine at concentrations of 0.72, 1.4 and 2.9 mg/ml supported the optimal population growth in *C. elegans*. There was no significant difference ($p < .05$) in the population growth at these levels.

Our study showed that the growth of *C. elegans* in L-forms of homocysteine was much more significant than D-forms. Among the methionine precursors tested, L-homocysteine thiolactone·HCl supported 46% population growth of nematodes compared to control group L-methionine (100%), whereas D-homocysteine thiolactone·HCl supported only 9.3%, and DL-homocysteine had less than 1% growth.

Based on our studies, the multiple metabolic blockage(s) in methionine biosynthesis may have occurred between homoserine and homocysteine, but the final blockage step occurred between cystathionine and homocysteine. The multiple metabolic blockage(s) for leucine may have occurred between α-ketoisovaleric acid and leucine, but the final metabolic blockage step occurred between α-ketoisocaproic acid and leucine.

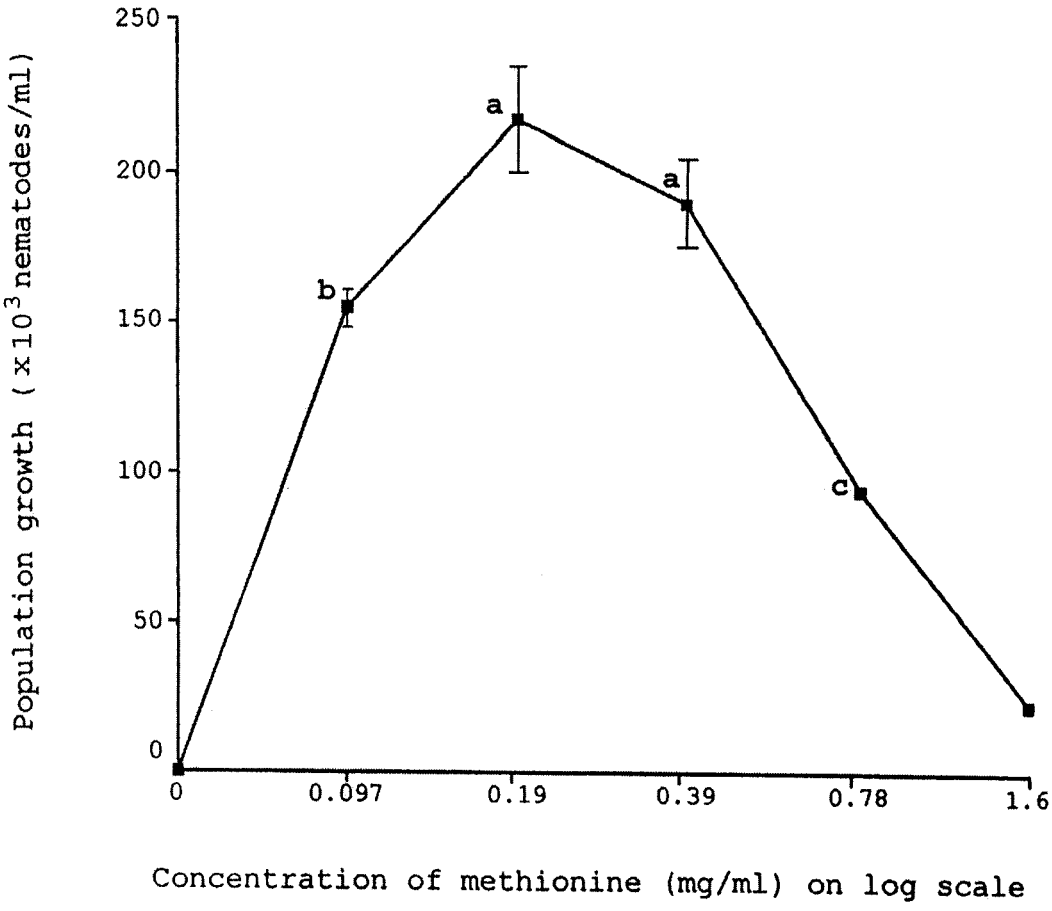

Figure 1. Dose response of *C. elegans* at various concentration of methionine in *Caenorhabditis elegans* Maintenance Medium. A total of 520 nematodes per ml were inoculated in each tube and the population growth was determined 23 days after inoculation. Bars represent ± standard deviation of the mean of four replicates; a, b, c = significantly different at $p < .05$

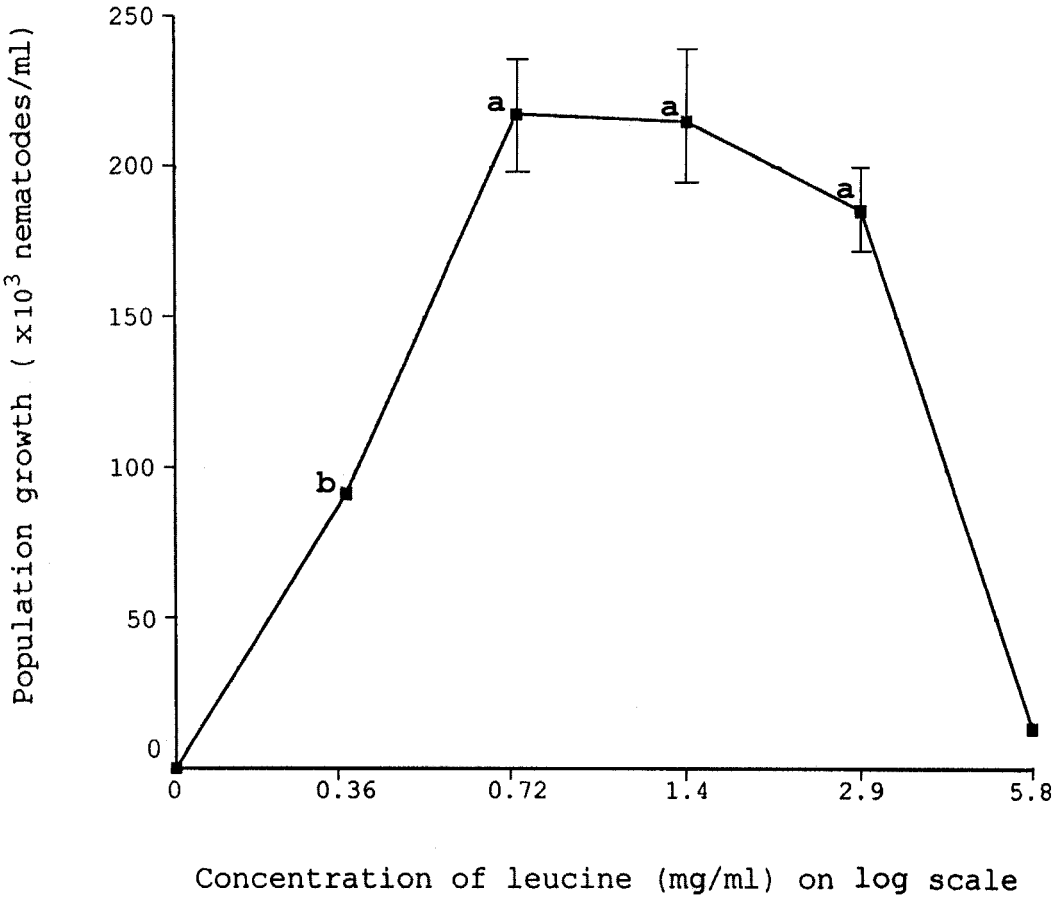

Figure 2. Dose response of *C. elegans* at various concentration of leucine in *Carnorhabditis elegans* Maintenance Medium. A total of 520 nematodes per ml were inoculated in each tube and the population growth was determined 23 days after inoculation. Bars represent ± standard deviation of the mean of four replicates; a, b = significantly different at $p < .05$

Table 2.1. Population growth of *C. elegans* with precursors of methionine

Precursors[a]	**mmole**	**Concentration (mg/ml)**	**Population**[b] **(nematodes/ml x10^3)**	**Percentage population growth of control**
Homoserine	2.61	0.31	0.43 ± 0.0	<1%
O-succinyl-homoserine	2.61	0.57	0.53 ± 0.0	<1%
Cystathionine	2.61	0.58	0.75 ± 0.10	<1%
L-homocysteine thiolaction·HCl	2.61	0.40	93 ± 1.9	46%
D-homocysteine thiolactone·HCl	2.61	0.40	19 ± 2.6	9.3%
DL-homocysteine	2.61	0.35	1.0 ± 0.13	<1%
L-Methionine (control)	2.61	0.39	203 ± 13	100%

[a]the precursors were supplemented at equimolar concentrations of the optimal level of methionine established in Fig. 1 (0.39 mg/ml or 2.61 mmole) in a basal media without methionine (CeMM minus methionine).

[b]data represent mean ± standard deviation.

Table 2.2. Population growth of *C. elegans* with precursors of leucine

Precursors[a]	**mmole**	**Concentration (mg/ml)**	**Population**[b] **(nematodes/ml x10^3)**	**Percentage population growth of control**
α-ketoisovaleric acid	10.7	1.5	0.10 ± 0.0	<1%
2-isopropylmalic acid	10.7	1.9	0.050 ± 0.0	<1%
α-ketoisocaproic acid	10.7	1.7	0.30 ± 0.0	<1%
L-leucine (control)	10.7	1.4	223 ± 9.6	100 %

[a]the precursors were supplemented at equimolar concentrations of the optimal level of leucine established in Fig. 2 (1.4 mg/ml or 10.7 mmole) in a basal media without leucine (CeMM minus leucine).

[b]data represent mean ± standard deviation.

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CHAPTER 3

SUMMARY AND RECOMMENDATIONS

Summary

The requirements of methionine and leucine were determined in the free-living nematode, *Caenorhabditis elegans* (*C. elegans*). The growth-promoting activities of six different concentrations of methionine (0.0, 0.097, 0.19, 0.39, 0.78 and 1.6 mg/ml) and leucine (0.0, 0.36, 0.72, 1.4, 2.9 and 5.8 mg/ml) were determined. Optimal population growth of *C. elegans* occurred at concentrations of 0.19 and 0.39 mg methionine per ml of medium, and 0.72, 1.4 and 2.9 mg leucine per ml of medium individually. The site of blockage in the biosynthetic pathway of methionine and leucine was determined by supplementing the precursors of these amino acids in equimolar concentrations of the optimal concentration established in the requirement experiments earlier. It was concluded that the metabolic blockage for methionine synthesis occurred between cystathionine and homocysteine, since L-homocysteine thiolactone hydrochloride had 46% efficiency and D-homocysteine thiolactone·HCl had 9.3% efficiency as methionine substitutes. The metabolic blockage of leucine occurred between α-ketoisovaleric acid and leucine, since none of the precursors tested supported growth in the nematodes.

Recommendations

To improve the experimental design of this study, the following recommendations are made.

1. More culture tubes (6-8) should be cultivated per concentration level of methionine and leucine, to increase the sample size for statistical analysis.
2. To determine the other possible limiting factors (e.g., absorption) in the conversion of homocysteine to methionine in *C. elegans*, the growth-promoting effect of L-homocysteine thiolactone hydrochloride (HCl) and D-homocysteine thiolactone·HCl should be increased to 2X (g/l) concentration, in order to determine the replacement efficiency compared to L-methionine.

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Appendix

COMPONENTS OF *CAENORHABDITIS ELEGANS* MAINTENANCE MEDIUM[a]

I. Vitamins & Growth Factors	MW[b]	gm[c]
A. Water Soluble (WSV)		
N-Acetylglucosamine	222.2	0.01500
Cyanocobalamine	135.4	0.00375
Niacinamide	122.1	0.00750
Pantethine	554.7	0.00375
Pantothenate (Ca)	238.3	0.00750
Pyridoxamine·2HCl	241.1	0.00375
Pyridoxine·HCl	205.6	0.00750
Pyridoxal·PO_4	247.1	0.00375
Riboflavin-5´-PO_4 (Na)·$2H_2O$	514.4	0.00750
Thiamin·HCl	337.3	0.00750
B. TEA Soluble (TEA)		
Biotin	244.3	0.00375
Niacin	123.1	0.00750
Pterolylglutamic Acid	441.4	0.00750
DL-Thioctic Acid	206.3	0.00375
p-Aminobenzoic Acid	137.1	0.00750
II. Salts		
$CaCl_2·2H_2O$	147.0	0.2205
$CuCl_2·2H_2O$	170.5	0.0065
$MnCl_2·4H_2O$	197.9	0.0222
$ZnCl_2$	136.3	0.0102
KH_2PO_4	136.1	1.2255
K_3 Citrate·H_2O	324.4	0.4860
Fe $(NH_4)_2(SO_4)_2·6H_2O$	392.2	0.0588
Mg $(OH)_2$	58.3	0.1740
Citric Acid·H_2O	210.1	0.6303

Appendix (continued)

III. Amino Acids	MW[b]	gm[c]
A. Essential Amino Acids (EAA)		
L-Arginine	174.2	0.9750
L-Histidine	155.2	0.2830
L-Lysine·HCl	182.6	1.2830
L-Tryptophan	204.2	0.1840
L-Methionine	149.2	0.3890
L-Threonine	119.1	0.7170
L-Leucine	131.2	1.4390
L-Isoleucine	131.2	0.8610
L-Valine	117.1	1.0200
L-Phenylalanine	165.2	0.6230
B. Non-essential Amino Acids (NEA)		
L-Phenylalanine	165.2	0.1800
L-Tyrosine	181.2	0.2720
L-Alanine	89.1	1.3950
L-Aaspartic Acid	133.1	1.6200
L-Cysteine·HCl·H_2O	175.6	0.0280
L-Glutamate (Na)·H_2O	187.1	0.5500
L-Glutamine	146.2	1.4630
L-Glycine	75.1	0.7220
L-Proline	115.1	0.6530
L-Serine	105.1	0.7880
IV. Nucleic Acid Substituents		
Adenosine-3′-(2′)-Phosphoric Acid·H_2O	365.2	0.3652
Cytidine-3′-(2′)-Phosphoric Acid	323.2	0.3232
Guanosine-3′-(2′)$PO_4(Na)_2$·H_2O	425.2	0.3632
Uridine-3′-(2′)-Phosphoric Acid	324.2	0.3242
Thymine	126.1	0.1261

Appendix (continued)

V. Other Growth Factors (GF)	MW[b]	gm[c]
Glutathione, reduced	307.3	0.2040
Choline H_2 Citrate	295.3	0.0885
Myo-Inositol	180.2	0.0645
Cytochrome c	12384.0	0.0500
β-Sitosterol	414.7	0.0500
VI. Energy Source		
D-Glucose	180.2	32.500
or K-Acetate[d]	98.1	5.0000
VII. Solvents		
KOH	56.1	[e]
Triethsnolsmine (TEA)	149.2	0.0325
Tween 80	1308.0	1.2500

[a]Lu and Goetsch, 1993.
[b]Molecular weight
[c]gm/500 ml (2X)
[d]A minimal 1.3 gm/500 ml glucose is also included.
[e]Needed for adjustment of pH to 5.9 ± 0.1